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Molecular quantitation and characterization of *Vibrio cholerae* from different seafood obtained from wetmarket and supermarket

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Abstract: *Vibrio cholerae* still represents a significant threat to human health worldwide despite the advances in hygiene, consumer knowledge, food treatment and food processing. In Malaysia, statistics in year 2009 have shown that among the food and water borne diseases, food poisoning has the highest incidence rate of 36.17 per 100,000 populations and with a mortality rate of 0.01 per 100,000 populations. In this study, 22 seafood samples comprising of fish, squid, crustacean and mollusks purchased from wet market and supermarket were analyzed. The Most Probable Number (MPN) and real time PCR was used to enumerate the *Vibrio cholerae* in seafood sample. The results showed that MPN-real time PCR of the samples from wet market had a maximum of >1100 MPN/g compare to 93 MPN/g enumerated from the MPN plate. The MPN-real time PCR in the samples from supermarket indicated 290 MPN/g as compared to 240 MPN/g enumerated from the MPN plate. The standard curves showed that there was a good linear correlation between the Ct values. The minimum level of detection of *Vibrio cholerae* standard DNA at targeted gene was 3 x 10^{-5} ng/µl.

Keywords: *Vibrio cholerae*, seafood, most probable number (MPN), Real Time PCR, wet market, supermarket

Introduction

Seafood products are of great importance for human nutrition worldwide. Approximately 90% of global aquaculture production is based in Asia. Potential food safety hazards of seafood products vary according to the culture system and may include foodborne trematode infections, foodborne disease associated with pathogenic bacteria and viruses, veterinary drug residues, and contamination by agrochemicals and toxic metals (Feldhusen, 2000).

There are at least ten genera of bacterial pathogens that have been implicated with seafood-borne diseases. Among them are bacteria that hail from the *Vibrioanaceae* family. The genus Vibrio of the family *Vibrioanaceae* embraces more than 60 species, mostly marine in origin and its taxonomy is continuously being revised due to the addition of new species (Etinosa *et al*., 2008).

In the early 1980s, evidence accumulated showed clearly that pathogenic Vibrio species, including *Vibrio cholera* are naturally originated as an estuarine bacterium occurring in the aquatic environment (Colwell, 1996). *Vibrio cholerae* non-O1/non-O139 is recurrently isolated from aquatic environments and in seafood. In Malaysia, cholera is described as a public health problem due to its sporadic outbreaks that occurs periodically (Vadivelu *et al*., 2000). Studies have also affirmed that the recruitment of foreign labour force from other cholera-endemic countries may foster the migration of strains into Malaysia, thus causing further outbreaks (Iyer *et al*., 2006). Despite the advances in hygiene, consumer knowledge, food treatment and food processing, this foodborne pathogens still represent a significant threat to human health worldwide.

In Malaysia, statistics in year 2009 have shown among the food and water borne diseases, food poisoning has the highest incidence rate of 36.17 per 100,000 populations and with a mortality rate of 0.01 per 100,000 populations. Cholera cases incidence rate have increased from the year 2008 to 2009 at 0.34 per 100,000 populations to 0.98 per 100,000 populations and with a mortality rate of 0.01 per 100,000 populations (Malaysia Health Fact, 2009). To date, there is limited studies reported in Malaysia that might lead to significant indication of *Vibrio cholerae* and *Vibrio parahaemolyticus* prevalence in foods, environment and clinical samples (Yuherman *et al*., 2000; Chen *et al*., 2004; Tanil *et al*., 2005; Lesley *et al*., 2005; Suzita *et al*., 2010).

The application of the conventional method is very useful in isolating *Vibrio cholerae*. Selective agar such as Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar and enrichment procedures are commonly used to detect *Vibrio cholera* in seafood as recommended by Food and Drug Administration (FDA). FDA has also described a three-tube MPN method, with selective enrichment broths for enumerating *Vibrio cholerae* in seafood samples (Kaysner *et al*., 2004). The

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enumeration is further enhanced with various PCR-fingerprinting techniques and molecular methods to detect *Vibrio cholerae*.

The recent FDA Bacteriological Analytical Manual (BAM) recommends enrichment of 25 g of raw oysters at 42 °C for the recovery of *Vibrio cholerae* (Kaysner *et al*. 2004) followed by screening of *Vibrio cholera*e with a conventional PCR such as multiplex PCR assay targeting various toxigenic genes. Multiplex PCR assays were successfully applied for the characterization and differentiation of different *Vibrio cholerae* strains or isolates (De *et al*., 2001; Mitra *et al*., 2001).

The detection method is further extended with the usage of molecular approach such as real time PCR technique. Real time PCR has the potential to provide a more sensitive design for the detection of diverse range of microorganisms with faster and reliable results (Gubala, 2006). SYBR Green I, TaqMan probe and molecular beacon are among the widely used fluorescents in real time PCR applications of environmental samples. The dual-labelled hydrolysis probe assay utilizes the 5′ end fluorophore to hydrolyze an internal probe labelled with a fluorescent reporter dye (HEX (hexacholoro-6-carboxyfluorescein) and a quencher dye. The probe is designed to hybridize to the DNA sequence between the PCR primers. During PCR amplification, cleavage of the dual-labelled hydrolysis probe separates the reporter dye and quencher dye, which results in increased fluorescence. Real-time PCR eliminates the need for subsequent PCR product verification that is required by other PCR amplifications, thereby reducing the amount of time needed for sample analysis (Lyon, 2001).

The objectives of this study were to quantify and characterize Vibrio cholerae in seafood samples besides to compare the conventional enumeration techniques (MPN) and MPN-Real Time PCR. Thus, this study demonstrated the use of MPN and real time PCR in detecting Vibrio cholerae from seafood obtained from wet market and supermarket.

Materials and Methods

Samples

A total of 22 types of seafood samples were randomly purchased from January 2010 to April 2010 at local supermarkets and wet markets in Malaysia as presented in Table 1. All the samples were categorized into 4 major groups of fish, squid, crustacean and mollusks. The samples were transported in portable coolers at ambient temperature and were processed immediately on arrival at the laboratory. 10 grams of the sample were weighted and were transferred into a sterile stomacher bag with 90ml of alkaline peptone water broth (APW) to obtain a 1: 10 dilution. The bags were subjected further for homogenization for 60s using a stomacher.

Table 1. Local seafood sample used in this study

Category	Sample (Local Name)	Scientific nomenclature	Wet market	Supermarket
			Code	Code
Fish	Ikan Sardin	*Sardinella* spp.	PS	TS
	Ikan Selar Kuning	*Selaroides leptolepis*	PSK	SSK
	Ikan Kembong	*Rastrelliger* spp.	PK	SK
Squid	Sotong Biasa	*Loligo* spp.	PSS	SS
	Sotong Katak	*Sepia* spp.	PSC	SC
Crustacean	Udang Putih Kecil	*Metapeneopsis lysianassa*	PP	SP
	Udang Putih	*Penaeus merguiensis*	PH	SH
	Udang Galah	*Macrobrachium rosenbergii*	PG	SG
	Udang Harimau	*Penaeus monodon*	PR	SR
Mollusk	Lala	*Obicularia obiculata*	PL	SL
	Kerang	*Anadara granosa*	PM	SM

Most Probable Number (MPN)

The method of Yamamoto *et al*. 2008 and the protocol described by Bacteriological Analytical Manual standard manual (Kaysner *et al*., 2004) were adapted with minor modification. Enrichment of culture using alkaline peptone water broth and isolation using Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar were shown useful by FDA, 2004. 10g of the meat was aseptically removed from the samples and placed into a sterile stomacher bag. 90ml of alkaline peptone water (1% peptone [Difco], 1% NaCl, pH 8.6) was added and the mixture in the bag was homogenized using a stomacher for 60 seconds. A three-tube MPN procedure as described in the standard procedure. Ten ml, 1 ml, 0.1 ml, 0.01 ml and 0.001 ml equivalent of the supernatant was inoculated into 10 ml of alkaline peptone water. 1 ml of the inoculation equivalents were transferred into 9 ml of fresh alkaline peptone water in triplicate. Inoculated tubes were incubated overnight at 42°C. 100 µl of the overnight culture from each respective tube was platted on Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar. The agar plates were incubated

overnight at 37°C. Presumptive single colonies of *Vibrio cholerae* was picked randomly and kept in semisolid agar for further analysis.

Boiled-cell DNA extraction method

Briefly, the overnight culture mixture was pelleted twice with 1 mL of the culture suspension centrifuged at 10,000 rpm for 5min. The supernatant were removed and 1 mL of sterile ultrapure water is added and vortexed. Next, the microcentrifuge tubes were subjected to boiling at 100°C for 7 min and immerged onto ice for 5 min immediately. Then the tubes were centrifuged at 10,000 rpm for 1 min. The supernatant were transferred into a new 1.5 mL microcentrifuge tube and used as template for real time PCR analysis.

Real time PCR analysis

The real time PCR cycling protocol and reaction component concentrations were optimized for detection of extracellular secretion protein (epsM) gene. The 20 µL final reaction contained the following: 10X PCR Buffer (Intron); 2.5 mM dNTP (Intron); 0.2 units I-Taq DNA polymerase (Intron); 250 nM sense (5′-GCTGACGGTACTCGTATCG-3′) and anti-sense (5′-TTCGGTTTGTAATCGTGCTTGAG-3′) primers generating a 88 base pair amplicon within the extracellular secretion protein (epsM) gene; 100 nM probe (5′HEX-CGGTACGCTCACTCAAAGCTGCC-BHQ3′) and 1 µl of boil-cell template. Real time PCR thermal cycling was run using the Rotor-Gene 6000 system (Corbett Research, Australia) utilizing an initial denaturation and polymerase activation step at 95°C for 3 min, followed by 40 cycles of denaturation for 95°C for 10 s, and a combined annealing and extension step at 60°C for 30 s with the instrument optics on.

Standard curve and sensitivity for Real Time PCR assay

A standard curve was obtained by analyzing a 10-fold serial dilution of *Vibrio cholerae* standard strain DNA. The extracted and quantified DNA was diluted to achieve 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 and 10^{10} genomic equivalents and amplified using the reaction conditions and cycling parameters described above. Samples were analyzed in triplicate. The DNA concentration was converted to genomic equivalents based on the assumption that single copies of the target sequence were present in the genome. A PCR mixture without DNA was used as a negative control (NTC).

The standard curve was generated by plotting the log value of the calculated colony forming units (CFU) per reaction versus the cycle threshold (Ct). The Ct is defined as the cycle where the fluorescent signal is sufficient to cross a defined threshold.

Results and Discussion

MPN analysis

In the case of analyzing Vibrio species from seafood samples, the Most Probable Number (MPN) method has proven to be effective with reliable results. During the MPN-Plate process, Vibrio cholerae colonies were well differentiated from the other Vibrio species morphology by using chromogenic agar medium Thiosulphate Citrate Bile Salt Sucrose (TCBS). *Vibrio cholerae* exhibits a distinguish yellow colour colonies on the TCBS agar medium.

The MPN-Plate from all the 11 wet market samples exhibits the presences of *Vibrio cholerae* colonies. Where else, only 22.7% or 5 samples from the 11 supermarket samples exhibit the presences of *Vibrio cholera*e colonies. The density of *Vibrio cholerae* in seafood samples was summarized in Table 2. The total number of Vibrio cholerae in the 22 samples was analyzed and resulted in wide range of <3 to >1100 MPN/g. The MPN/g estimate of *Vibrio cholerae* in the samples from wet market had a minimum of 23 MPN/g and maximum of 93 MPN/g. The MPN/g estimation of *Vibrio cholerae* in the samples from supermarket had a minimum of <3 MPN/g and maximum of 240 MPN/g.

The occurrence of *Vibrio cholerae* in various seafood samples is probably a reflection of the atmosphere at the supermarket and wet market. The contamination could occur due to the way of handling the seafood at wet markets, which is less hygienic compared to supermarkets. From our scrutiny at the sampling site, the seafood is handled in hygienic manner. At the supermarkets, their daily routine practice requires the samples to be first washed and packed before being sold. Although it is handled in hygiene manner, the samples do contain Vibrio cholerae because it could be due to possible cross-contamination occur during the handling process. This is well supported by researchers (Chai *et al*., 2008; Yang *et al*., 2008; Tunung *et al*., 2010) who have reported that mishandling and poor hygiene is the main cause of cross-contamination of food at supermarkets.

The results emphasize the fact that seafood could be contaminated with *Vibrio cholerae* and thus could

The main advantage of the real time PCR is that it is very rapid and is a valuable method for screening a large number of samples. The real value of the real-time PCR is the potential for rapid analysis of

Table 2. MPN-Plate and MPN-Real Time PCR of *Vibrio cholerae* from wet market and supermarket

Sample	MPN-qPCR				MPN-PLATE			
	First Set	Middle Set	Last Set	MPN/g	First Set	Middle Set	Last Set	MPN/g
Wet market								
PS	0	0	0	<3	3	0	1	38
PSK	3	1	2	120	3	2	0	93
PK	2	1	2	27	3	0	0	23
PSS	3	3	3	>1100	3	2	0	93
PSC	3	0	3	95	3	2	0	93
PP	3	2	2	210	3	0	0	23
PH	3	2	2	210	3	2	0	93
PG	1	0	1	7.2	3	2	0	93
PR	3	2	2	210	3	0	0	23
PL	3	3	3	>1100	3	2	0	93
PM	2	3	1	36	3	0	0	23
Supermarket								
TS	3	2	3	290	3	2	1	150
SSK	1	0	1	7.2	0	0	0	<3
SK	1	2	0	11	0	0	0	<3
SS	3	2	3	290	0	0	0	<3
SC	0	0	0	<3	3	0	0	23
SP	3	0	1	38	3	0	0	23
SH	0	0	0	<3	0	0	0	<3
SG	3	1	0	43	3	0	0	23
SR	0	0	0	<3	0	0	0	<3
SL	0	2	0	6.2	0	0	0	<3
SM	3	2	3	290	3	3	0	240

possibly act as a transmission vehicle. The detection of *Vibrio cholerae* in all the samples is of concern, however this concern is largely alleviated because the concentration of *Vibrio cholerae* was low with a minimum of <3 MPN/g. The only disadvantage of the MPN method combined with conventional confirmation techniques were usage of media plates, the workload and the time needed to complete the identification (usually 4 to 7 days). However with the combination of specific molecular techniques enables the completion of enumeration in approximately 2 days (Martin *et al*., 2004).

Real Time PCR analysis

The study has successfully applied MPN-real time PCR for the enumeration of *Vibrio cholera*e in various seafood samples. The employment of this highly sensitive real time PCR method is appeared to be useful for rapid and quantitative detection of *Vibrio cholerae*. Previous researcher Nogva *et al*., 2000 has employed a highly sensitive and quantitative method dual-labeled hydrolysis real time PCR that is useful for estimating the number of cells of a specific pathogen in a food product. In this study, the method has allowed the determination of *Vibrio cholerae* occurrence in 22 seafood samples from wet market and supermarket. The density of *Vibrio cholerae* in seafood samples was summarized in Table 2. From the analysis, the samples yielded in a range widely from <3 to >1100 MPN/g. The MPN/g estimate of *Vibrio cholerae* in the samples from wet market had a minimum of <3 MPN/g and maximum of >1100 MPN/g. The MPN/g estimate of *Vibrio cholerae* in the samples from supermarket had a minimum of <3 MPN/g and maximum of 290 MPN/g.

The usage of real time method is well supported by Lyon *et al*., 2001 in which reported the usefulness of real time PCR to detect *Vibrio cholerae hlyA* gene in raw oyster and seawater, regardless of serotype and possession of the *ctx* gene. Previous study has also reported that real time PCR was a tool in enumerating *Vibrio cholerae* in seafood (Blackstone *et al*., 2007).

numerous pathogen-free samples, thereby allowing laboratories the ability to quickly screen products before they are released for human consumption. Putative positive food samples can also be quickly identified and pulled for further analysis (Lyon, 2001).

Standard curve and sensitivity detection

The minimum level of detection of Vibrio cholerae standard DNA at targeted gene was $3x10^{-5}$ ng/ul with a Ct value of 36.61. The average Ct values for each concentrations of DNA are shown in Table 3. As the concentration of DNA in a reaction mixture decrease, the Ct value increases (Table 3). The detection limit of *Vibrio cholerae* standard DNA is at 3x10-5 ng/ul. The standard curves showed that there was a good linear correlation between the Ct values. The standard curve slope was –3.469 with the reaction efficiency of 94%.

Table 3. Sensitivity of detection of *Vibrio cholerae* standard DNA by using real time PCR and probe.

Samples	Amount of DNA ng/ul	*Ct* Value
A	3×10^{-1}	22.99
B	3×10^{-2}	26.17
C	3×10^{-3}	29.54
D	3×10^{-4}	34.78
E	3×10^{-5}	36.61
F	3×10^{-6}	
G	3×10^{-7}	
H	3×10^{-8}	
I	3×10^{-9}	
J	3×10^{-10}	

Conclusion

In conclusion, the study revealed that MPN-Real time PCR is a useful tool for detection *Vibrio cholerae* in seafood. Although the contamination of *Vibrio cholerae* in seafood is low, it should not as a reason to ignore the bacteria contamination. The usage of powerful new methods such as real time PCR has given more complete understanding of the distribution and ecology of pathogenic Vibrios in the environment and food supply. This knowledge is a great aid for current efforts to assess and control the risk that these organisms present to public health (Nishibuchi *et al*., 2005). Early monitoring of *Vibrio cholerae* is important in preparation of risk assessment plan relating to seafood.

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